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THROMBOPOIETIN (c-mpl LIGAND) ACTS SYNERGISTICALLY WITH ERYTHROPOIETIN AND STEM CELL FACTOR TO ENHANCE MURINE MEGAKARYOCYTE COLONY GROWTH AND INCREASES MEGAKARYOCYTE POLYPLOIDY *in vitro*. V.C. Broudy, N. Lin,* and K. Kaushansky. University of Washington, Seattle, WA.

Thrombopoietin (Tpo), the ligand for the c-mpl receptor, is a major regulator of platelet production *in vivo*. Treatment of mice with purified recombinant Tpo increases platelet count four-fold, and expands CFU-meg numbers. Because other cytokines including IL-3, IL-6, IL-11, Epo, and SCF can stimulate megakaryopoiesis, we examined the effects of Tpo in combination with these cytokines on megakaryopoiesis *in vitro*. Recombinant Tpo, expressed by BHK cells, was provided by ZymoGenetics, Inc. Murine marrow cells were cultured in agar in IMDM supplemented with 10% horse serum and β-mercaptoethanol in the presence of recombinant growth factors, and CFU-meg were counted on day 5. Megakaryocyte ploidy was analyzed using murine marrow cells cultured for 5 days in IMDM supplemented with 1% nutridoma and recombinant growth factors. Megakaryocytes were identified by labeling with the 4A5 antibody and ploidy was analyzed by flow cytometry.

	#CFU-Meg/2.5 x10^5 Marrow Cells*			
Conc. Tpo	Tpo alone	+Epo (2U/ml)	+SCF (50 ng/ml)	+IL-3 (20 ng/ml)
0	0	0.3	5.7	20.3
5 U/ml	4.3	15.0	21.3	33.7
17 U/ml	13.3	41.7	35.0	47.0
50 U/ml	13.7	45.3	36.7	48.7
230 U/ml	23.0	54.3	47.3	58.7
670 U/ml	29.3	54.3	57.7	62.0

*mean of triplicate plates from 1 of 3 similar experiments.

IL-3 alone supported CFU-meg colony growth, and the effects of Tpo plus IL-3, Tpo plus IL-6 and Tpo plus IL-11 appeared to be approximately additive, in contrast to the effects of Tpo plus Epo or Tpo plus SCF. Fifty percent of megakaryocytes generated in cultures containing IL-3, SCF, or Epo displayed ≤ 8N ploidy. In contrast, cultures containing Tpo uniquely generated large numbers (30-35%) of megakaryocytes with ≥ 64N ploidy, and megakaryocytes with a median ploidy of 16-32N. These results demonstrate that an early acting multi-potent cytokine (SCF) as well as a late acting erythroid cytokine (Epo) can synergize with Tpo to stimulate proliferation of CFU-meg.

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EFFECTS OF RECOMBINANT HUMAN THROMBOPOIETIN (rhTPO) ON COLONY FORMATION BY HUMAN HEMATOPOIETIC STEM CELLS. Lawrence A. Solberg, Jr., Detra Wilson*, Frederick de Sauvage*, and Dan Eaton*. Division of Hematology-Oncology Mayo Clinic Jacksonville, Jacksonville, FL and Genentech, Inc., South San Francisco, CA

Full-length recombinant human thrombopoietin (rhTPO) and the N-terminal 153 amino acid residues (rhTPO$_{153}$) have been expressed by human embryonic kidney cells. The homology of rhTPO to EPO and the expression of the c-MPL hematopoietic receptor on megakaryocytes and CD34+ hematopoietic stem cells suggest that rhTPO and rhTPO$_{153}$ might stimulate progenitors committed to megakaryocytopoiesis but also might influence erythroid and multilineage progenitor cells. We are studying the effects of rhTPO and rhTPO$_{153}$ singly or in combinations with rhIL3, rhEPO, rhIL6, rhIL11, rhSCF, and rhGM-CSF on CFU-GM, CFU-MEG, BFU-E, and CFU-GEMM derived colony formation in methylcellulose cultures. Stem cells present in mononuclear (MNC) and CD34+ cell populations from human peripheral blood (PB) and bone marrow are being used. Representative results here are expressed as mean colony counts ± SEM per 400,000 PB MNC for n=4 and growth factors as units or ng per mL. rhTPO and rhTPO$_{153}$ singly and in combination with rhIL3 (0.8 ng) resulted in a dose-dependent increase in CFU-MEG colonies, e.g., 27±8 and 18.5 ± 4.3 with 2 U and 49.5 ± 9.8 and 30 ± 7.3 with 7.5 U, respectively. Addition of rhIL3 to rhTPO and rhTPO$_{153}$ increased CFU-MEG colony counts slightly compared to those with rhTPO or rhTPO$_{153}$ as single agents, but the increases did not achieve statistical significance. BFU-E formation with rhEPO 0.3 U was 69± 20; with rhIL3 and rhEPO 134±8; with 5U rhTPO 7.5±4 ,5U rhTPO and rhIL 129±36; 5U rhTPO$_{153}$ 24.5±9, 5U rhTPO$_{153}$ and RHIL3 77±44. rhTPO and rhTPO$_{153}$ minimally increased BFU-E formation added alone but synergized with rhIL3. All BFU-E colonies grown with rhTPO or rhTPO$_{153}$ were smaller and less well hemoglobinized than colonies grown in the presence of rhEPO. rhTPO and rhTPO$_{153}$ also stimulated CFU-GEMM derived colony formation with rhIL3, rhEPO, and rhSCF being synergistic. No effects were seen on CFU-GM derived colonies.

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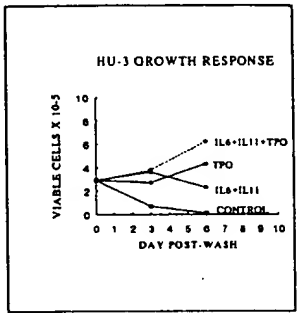
DIFFERENTIAL EFFECTS OF THROMBOPOIETIN (MPL) ON CELL LINES MB-02 AND HU-3 DERIVED FROM PATIENTS WITH MEGAKARYOBLASTIC LEUKEMIA. D. Morgan*, G.Soslau, and I.Brodsky Hahnemann University, Philadelphia, PA.

MB-02 and HU-3 are leukemia-derived cytokine-dependent cell lines with erythroid and megakaryoblastic potential (Blood 82:373a, 1993). Long-term growth of MB-02 requires GM-CSF while HU-3 responds equally to GM-CSF or IL-3. HU-3 is further along the megakaryocytic pathway by the constitutive expression of surface GpIIb/IIIa which is detected on MB-02 only after phorbol ester induction. Membrane GpIb is not present nor induced by phorbol esters on either cell line.



Recombinant thrombopoietin (TPO) (Zymogenetics, Seattle, WA) was assayed for the ability to induce megakaryocytopoiesis in MB-02 and HU-3 cells deprived of GM-CSF. TPO (400u/ml) alone and in synergy with IL-6 + IL-11 supported growth of HU-3 and induced the first-time detection of GpIb as measured by monoclonal antibodies and indirect immunofluorescence on fixed cell preparations. ***The bi-potential MB-02 cells failed to survive when cultured under the same conditions.*** Preliminary data from further studies on HU-3 suggest a synergy between TPO and erythropoietin (EPO). TPO induces GpIb when added to GM-CSF or IL-3 containing cultures, but does not enhance growth until EPO is added. As shown previously, EPO alone has no proliferative effect on HU-3. These early studies suggest that TPO can have a direct effect on the maturation of early megakaryocyte precursors, but may require other components of the bone marrow microenvironment before lineage commitment of the bi-potential progenitor can occur.

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PURIFICATION OF A MEGAKARYOCYTE GROWTH AND DEVELOPMENT FACTOR FROM APLASTIC CANINE AND PORCINE PLASMA. Y.S. Li,* P. Hunt, J. Bogenberger,* J. Nichol, A. Knudten,* J. Skrine,* L. Merewether,* C. Clogston,* S. Swift,* V. Parker,* R. Bosselman,* H. Lu,* T. Bartley.* Amgen, Inc. Thousand Oaks, CA.

The key regulator of megakaryocytopoiesis and platelet production has long been thought to be a lineage-specific cytokine. Here we report the complete purification of megakaryocyte growth and development factor (MGDF) from the aplastic canine and porcine plasma. The proteins were purified by sequential chromatography of wheat germ agglutinin (WGA), Mpl receptor affinity, ion-exchange, gel filtration and reversed-phase HPLC. At least two forms of purified canine MGDF, with apparent molecular weights of 25,000 and 31,000, were observed upon analysis by SDS-polyacrylamide gel electrophoresis (PAGE), and they share an identical amino-terminal sequence, APPAXDPRLLNKMLRDSHVLH. The purified porcine MGDF also revealed the presence of several forms by SDS-PAGE analysis. The major form of purified porcine MGDF has an apparent molecular weight of 17,000 with an amino-terminal sequence of SPAPPA(X)DPRLLNKLLRDSHVLHGR, homologous to the canine form. All purified MGDF supported the development of megakaryocytes from human CD34+ progenitor cell populations in liquid culture, and also promoted the survival of a factor-dependent murine cell line (32D) engineered to express the putative cytokine receptor, Mpl. The *in vitro* activities of MGDF were blocked by the addition of the soluble, extracellular domain of Mpl. These results suggest that MGDF is the ligand for Mpl, and a specific cytokine for the megakaryocyte lineage.

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RECOMBINANT THROMBOPOIETIN STIMULATES RAPID PLATELET RECOVERY IN THROMBOCYTOPENIC MICE. K.H. Sprugel*, J.M. Humes*, A. Grossmann*, H. P. Ren* and K. Kaushansky. ZymoGenetics, Inc, and Division of Hematology/Dept. of Medicine, University of Washington, Seattle, WA.

The recent cloning of thrombopoietin has prompted speculation that it could be used clinically to improve platelet counts in patients with decreased platelet production. This possibility was explored in a mouse model of bone marrow injury that results in a prolonged period of thrombocytopenia. Dose response studies were first performed in normal mice using recombinant mouse thrombopoietin (rmTPO). Mice were dosed daily with 12.5-100 kU rmTPO for 10 days and platelet counts were monitored. Circulating platelet counts increased 30% by day 3 and 2.5-4 fold by day 7. Platelet counts returned to normal by 14 days after cessation of treatment. To test the effects of TPO on platelet production in myelosuppressed animals, C57/Bl6 mice were administered a combined radiation/ chemotherapeutic regimen. Mice were irradiated with 500 cGy using a ^{137}Cs source and given a single dose of 1.2 mg carboplatin. By day 8, platelet counts dropped to $\leq$ 20% of baseline and remained at this level through day 14. Platelet counts returned to baseline between day 25 and day 27. Mice treated with 25 or 75 kU rmTPO/day for 18 days showed marked improvements in platelet recovery. The platelet counts in the 75 kU/day group returned to normal by day 11 while the 25 kU/day group was normal by day 13. This was an improvement of 16 and 14 days, respectively, relative to vehicle. The time to 50% recovery was similarly improved. In addition, mice treated with 75 kU rmTPO/day had twice as many circulating platelets at the nadir as the mice treated with 25 kU/day or vehicle. Circulating WBC counts recovered at the same rate in both rmTPO and vehicle treated mice. These experiments demonstrate that rmTPO, in the absence of other exogenous hematopoietic factors, can improve the rate of recovery of platelets in thrombocytopenic mice. This suggests that TPO may be useful clinically.

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THE PURIFICATION AND PHYSIOLOGICAL CHARACTERISTICS OF OVINE THROMBOPOIETIN. D.J. Kuter, D.L. Beeler,* and R.D. Rosenberg. Dept. of Biology, Massachusetts Institute of Technology, Cambridge, MA.

Megakaryocyte (MK) growth and subsequent platelet production have long been considered to be controlled by a humoral substance known as thrombopoietin (TPO). We have found that thrombocytopenic (TC) plasma stimulated an increase in MK ploidy in bone marrow culture and have used this assay to purify a molecule 9.4 million-fold from TC sheep plasma using standard protein purification methods. The amino acid sequence of the 31,200 Da protein is 74% identical to both human and murine c-Mpl ligand and binds to the c-Mpl receptor This purified ovine. TPO (o-TPO) doubled the number of MK in bone marrow culture and increased the average ploidy from 10.4 to 18.8. Upon injection into rats, o-TPO produced a 77% rise in the platelet count after 4 days and increased the average MK ploidy from 17 to 30. To prove that this molecule was the physiologically relevant TPO, the amount of TPO in the circulation was measured during thrombocytopenia. TPO was not elevated in the circulation 3 h after the onset of acute thrombocytopenia, but was half-maximal after 8 h. Levels of TPO were inversely proportional to the platelet count and rose from normal of less than 0.25 pM to 25-50 pM. Infusion of platelets into TC animals rapidly returned levels of TPO to normal and showed a half-life of less than 45 min for TPO. Since TPO was found to bind to platelets, we suggest that circulating levels of TPO are directly maintained by the platelet mass, obviating any need for a platelet "sensing mechanism."

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RECOMBINANT HUMAN MGDF (rhuMGDF), A LIGAND FOR c-MPL, PRODUCES FUNCTIONAL PLATELETS FROM MEGAKARYOCYTES IN VITRO. E. Choi,* J. Nichol,* M. Hokom,* A. Hornkohl,* and P.Hunt. Developmental Megakaryocytopoiesis, AMGEN Inc., Thousand Oaks, CA.

A recently described ligand for c-mpl, recombinant human Megakaryocyte Growth and Development Factor (rhuMGDF) was used to study the generation of platelets from megakaryocytes in vitro. CD34+ cells were isolated from leukapheresis units of normal donors with informed consent and were cultured in 20 ng/ml of rhuMGDF and 10 % human AB plasma in IMDM supplemented with 1X non-essential amino acids, 1X minimum essential medium vitamins, 1X sodium pyruvate, 0.2% deionized BSA, 2 µg/ml L-asparagine, 0.1 µM 3-mercapto 1-propanediol, and 1X penicillin-streptomycin-glutamine. After 8 days in culture, an average of 95 percent of the cultured cells expressed GPIb and IIb proteins, characteristic of megakaryocytes. After two additional days of culture, proplatelets, the cytoplasmic extensions of mature megakaryocytes and an antecedent structure of platelets, were observed. Further culturing of these proplatelets resulted in platelets which were morphologically and functionally identical to plasma-derived platelets. To study the functional aspect of these in vitro generated platelets, the platelets were collected and washed through a gel filtration column. PAC-1 (Shattil et al. 1985), a monoclonal antibody against the activated form of GPIIb/IIIa, was used to detect activated platelets. When in vitro generated platelets were stimulated with ADP, the presence of activated GPIIb/IIIa was significantly increased, as detected by flow cytometry. Other functional aspects of these rhuMGDF generated platelets will be discussed.

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BIOLOGICAL PROPERTIES OF THROMBOPOIETIN (TPO). H. Miyazaki*, K. Horie*, T. Tahara*, E. Maeda*, H. Akahori*, Y. Shimada*, K. Ogami*, H. Ohashi*, T. Ozawa*, A. Kokubo*, K. Kawamura*, T. Kato*. (Intr. by H. Hirai) Pharmaceutical Research Laboratory, Kirin Brewery, Co., Ltd., Maebashi, JAPAN.

We have recently purified and cloned thrombopoietin (TPO) and found that TPO has sequence identity to the Mpl ligand. In this investigation, we evaluated the effects of recombinant human TPO (rhTPO) on rat megakaryocyte progenitor cell (CFU-MK) growth in vitro, mainly using a GpIIb/IIIa+ subpopulation of bone marrow cells highly enriched for CFU-MK (about 10% purity). rhTPO alone exhibited substantial megakaryocyte colony stimulatory activity, inducing 42 colonies per 10^3 GpIIb/IIIa+ cells plated at an optimal concentration. The number of megakaryocytes per colony stimulated with rhTPO averaged 5.9, which was much lower than the value for rat IL-3. On the other hand, the average size of megakaryocytes in colonies stimulated with rhTPO was largest among the cytokines tested. Only megakaryocyte colonies were formed by both nonadherent bone marrow cells and GpIIb/IIIa+ cells in the presence of rhTPO. rhTPO stimulated a dose-dependent increase in the number and size of acethylcholinesterase+ cells and the incorporation ^{14}C-serotonin as a marker for megakaryocyte growth according to the culture period in liquid culture. On 4 days of culture, substantial number of megakaryocytes underwent dramatic morphological change, known as proplatelet formation. In addition, modal ploidy classes of megakaryocytes grown from GpIIb/IIIa+ CFU-MK with rhTPO were shifted to higher values according to the culture period. In vivo administration of rhTPO induced a marked increase in platelet counts in mice. These results suggest that TPO is a lineage-specific factor that stimulates CFU-MK to proliferate and differentiate into mature megakaryocytes which have the ability to produce platelets.